(12) UK Patent Application (19) GB (11) 2 253 859 (13) A

(43) Date of A publication 23.09.1992

(21) Application No 9104277.0

(22) Date of filing 28.02.1991

(71) Applicant
Microbial Developments Limited

(Incorporated in the United Kingdom)

Spring Lane North, Malvern Link, Worcestershire, WR14 1AH, United Kingdom

(72) Inventors
C A Day
B W Holton

(74) Agent and/or Address for Service
Marks & Clerk
57-60 Lincoln's Inn Fields, London, WC2A 3LS, United Kingdom

(51) INT CL[5]
A01N 63/00

(52) UK CL (Edition K)
C6F FE
U1S S1070 S1086 S1307

(56) Documents cited
EP 0414304 A2

(58) Field of search
UK CL (Edition K) **C6F FE FF FX**
INT CL[5] **A01N, C12N**
Online databases: WPI; BIOTECH (DIALOG)

(54) Use of bacteriophages to prevent microbial infestation

(57) Bacteriophages are used in areas where foodstuffs are prepared and stored to treat or prevent infestation by harmful microbes, e.g. Listeria monocytogenes or clostridia sp. in dairy environments. The phage may be applied as a spray at a concentration of about 10^2 to 10^{11} pfu.

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ENVIRONMENTAL SPRAYS

The present invention relates to preparations for the prevention and treatment of microbial infestations in food-stuffs.

Food-stuffs are susceptible to a wide variety of microbial infestations. These are rarely beneficial and frequently harmful. Those of a harmful nature can cause mild tainting of the food-stuff, or illness ranging from mild stomach ache to death in the consumer, always assuming that the food is not rendered completely inedible.

Milk products, and especially cheese, are particularly prone to infection by such organisms as Listeria, which is an opportunist pathogen, and Clostridia which can result in an unpleasant acid taste which may be combined with uncontrolled gas production to cause an unsightly, deformed product.

Microbial infestation need not always be undesirable. The production of certain cheeses, for example, is totally reliant on the presence of certain fungi, and yoghurt is a thick culture of harmless bacteria.

Listeria organisms are found effectively everywhere, such as in the soil, water and air. In dairy environments, such as in the manufacture of cheese, Listeria monocytogenes has been shown to be present in drains and on wet floors in preparation areas as well as in floor samples in the 'maturing' rooms.

Listeria organisms can also be a nuisance in the

manufacture of blue cheeses which, once made, are injected with specific fungal species (to make the 'blue' veining, for example, Stiltons) and this is a problem entry point for Listeria.

Further, the mixing of cheeses to make soft 'blended' varieties is particularly at risk from environmental Listeria, most of the contamination of these cheeses arising at the time of blending.

Normal disinfection of the dairies does not always eradicate all Listeria, particularly in inaccessible places, such as drains.

It has now been discovered that the use of bacteriophages in areas where foodstuffs are prepared and stored can prevent infestation by harmful microbes.

Thus, in a first aspect of the present invention, there is provided the use of at least one species of bacteriophage in an area where a foodstuff is prepared or stored to treat or prevent bacterial infection in the foodstuff.

The foodstuff may be any that is prone to infection by bacteria, and the bacteriophage may be used at any time from commencement of preparation of the foodstuff, for example, to final consumption. The foodstuff may be such as meat products, such as pate or sausages, or fish and/or vegetable preparations, for example. However, the use of the invention is generally preferred to be in connection with a dairy product, such as yogurt, but especially cheese.

The bacteriophage (also referred to as phage herein) may be used in any suitable manner to treat of prevent infestation. The phage may be applied as a solution or

dry powder, for example, to surfaces in the relevant areas, or may be applied as a spray or dust. A fine spray or other form which can be distributed in the atmosphere of the preparation or storage areas, without settling too rapidly, is particularly beneficial, in that this will allow the phage to spread out and to reach areas which cannot normally be reached directly, such as drains.

Distribution of a suitable phage preparation in the atmosphere or other parts of the preparation or storage environment may be by any suitable means. Industrial sprayers or aerosols may be employed to disperse a fine solution of the phage, and these may be aided, for example, by suitable means to agitate the air to further ensure adequate distribution of the phage.

Bacteria against which phages may be used in the use of the present invention may be any that infest foodstuffs. Clostridium and Listeria are general examples of harmful bacteria, with Listeria, especially L. monocytogenes, being a problem in the manufacture of cheeses. As used throughout this specification, where Listeria is used, this term also encompasses other bacteria which infect foodstuffs. Likewise, where the term cheese is used, other foodstuffs are also to be understood.

An environmental spray containing listeriaphages to reduce the incidence levels of Listeria in the atmosphere of cheese-making dairies is preferred, and an environmental spray may be used at any stage in the preparation of cheese, but particularly when there is risk of exposure to Listeria. Particular occasions where sprays may be used have been described above. For example, the spray may be used before or following normal disinfection, before the process of blending

cheeses, or before the injection of 'blue' fungi.

An environmental listeriaphage spray may be produced as a concentrated liquid or a soluble powder (say, in dextrose) - to be mixed with water and added to any propriety spraying equipment, at levels of 10^2 to 10^{11}.

The product may contain any number of phage types which are active against as many strains of Listeria, especially L. monocytogenes as possible, to increase the efficacy of the phage spray with respect to the reduction or elimination of as many strains as possible.

The present invention also provides preparations containing at least one variety of bacteriophage and a suitable carrier therefor for use as described.

Bacteriophages are highly host-specific. Extremely rapid multiplication within the host cell occurs leading to the destruction of the cell (lysis) and the release of up to 20,000 new phages, each capable of further infection. Phages are essentially non-living outside the host cell and, therefore, can exhibit very considerable longevity, making them particularly useful in preparations according to the present invention.

It will be appreciated that as bacteriophages are highly specific in the organisms they can infect, any one variety of phage will only infect one species of bacterium and, frequently, only selected strains of that species. There is thus no danger to the consumer of being infected by the phage.

A particular advantage in the use of phages according to the present invention is that, with only extremely small quantities of phage being required for

efficacy, there is no adverse effect on flavour, should the phage get into the foodstuff. Furthermore, as phages only infect highly specific organisms, no propagation of phage can take place in the absence of the host bacterium, so the cheese remains completely unaffected by their presence.

A disadvantage of the antibacterial treatments of the art generally lies in their inability to reach all the harmful bacteria. Such problems are further compounded by the requirement not to get into the foodstuff, as this may both have a deleterious effect on flavour and also kill bacteria or fungi which may be an important part of the foodstuff, or in its preparation. Phages do not suffer from these problems, as they may be freely dispersed in the preparation or storage areas and may be allowed to contact the foodstuff, being both tasteless and harmless. Phages provide the solution to the problem and can be selected against any bacterium as required.

Thus, in a further aspect of the present invention there is provided the use or process as defined above wherein the bacteriophage(s) is selected according to host bacterium specificity.

It will be appreciated that, while lysogenic phages can be used in accordance with the invention, the use of lytic phages is generally preferred, as infection results in the rapid destruction of the unwanted bacterial host.

Bacteria are known to be able to develop resistance to phage infection. The present invention, therefore, further provides a use or process as described above wherein the preparation comprises at least two strains of phage specific for one host. If the target

micro-organism develops a resistance to one phage, or the phage becomes lysogenic, elimination of the unwanted organism still occurs.

Still more preferable is the 'rotation' of phages. For example, in the case where three phages are available against Clostridia spp., then three preparations of different phage pairs are available for use in successive treatments to minimise the risk of resistance developing.

As used herein, 'rotation' means varying the bacteriophage composition of preparations according to the present invention with different batches of cheese prepared in the same locale. Such variation need not be cyclical, or even regular, provided that different compositions are used occasionally to prevent resistance developing.

Preparations according to the present invention may contain phages specific for several different species of bacterium. A suitable treatment may contain phages specific for two Clostridium and one Listeria species, for example.

Listeria spp. are particularly susceptible to treatment according to the present invention. Anaerobes are generally significantly less efficient than aerobes, as life processes must be restricted to essentials to allow effective exploitation of the anaerobic environment. Thus, while the likes of E. coli have highly sophisticated mechanisms against phage attack, anaerobes are generally less able to generate a defence.

Suitable bacteriophages for use according to the present invention may be isolated from natural sources, preferably using known bacteriophage enhancement

techniques [c.f. Betz, J.V., & Anderson, K.E., (1983), J. Bact., 87, 408]. Bacteriophages so isolated may be characterised as to their host specificity by known techniques.

Suitable quantities of phage for use according to the present invention may be obtained, for example, by a batch technique, wherein a culture of host bacterium is grown nearly to capacity and then seeded with phage. After a suitable time has elapsed to allow maximal phage propagation, the culture is further lysed by chemical or physical techniques, if required, and the lysate spun down. The phage-bearing supernatant may then be further purified, for example by ultrafiltration, and concentrated (freeze-drying, for instance). The resulting preparation can be used directly or further combined with other ingredients to aid in packaging, end-use etc.

Large-scale commercial production of Listeria-specific phages may involve initial anaerobic fermentation of the host Listeria species, preferably in optimal submerged culture conditions for a time adequate to achieve logarithmic growth of the culture. Specific phage preparations are then introduced to the culture and incubation continued until maximal lysis can be demonstrated. Downstream recovery of such a phage preparation from solution may be effected by initial low-speed centrifugation to remove any remaining bacterial cells and debris, and the phage purified and concentrated by ultracentrifugation and ultrafiltration techniques. The resulting concentrated phage preparation can be cryoprotected and freeze-dried by techniques well-known in the art, or preferably plated onto, or mixed with, a suitable carrier material and air- or vacuum-dried as appropriate. Bacteriophages can also be encapsulated in acid-resistant biodegradable

gums to provide a composite additive product optionally containing organic acids. Typical activity levels of phages prepared according to the above methods range from 10^7-10^{12} pfu/gram of concentrate according to the particular phage morphology.

Preparations according to the present invention suitably contain at least two varieties of phage, optionally specific for more than one family of bacterium, as required. The preparations may be liquid or solid according to requirements. Liquid preparations may be simple suspensions of phage in water, but preferably further comprise a suitable carrier. Suitable carriers may, for example be sugar-based, such as mannitol, but may comprise any suitable substance known in the art.

Liquid preparations may be prepared from any of the preparations generally available for similar use, a suitable quantity of phage being added. In the alternative, a more concentrated 'stock' solution may be prepared for later dilution.

In order to determine phage activity in the final product, as well as during preparation, total specific phage counts may be undertaken using, for example, the double agar layer plating technique for plaque production, employing the host microbial species in each case. The method described by Adams, M.H., in "Bacteriophages" (Interscience Publishers (1959)) is suitable for this purpose.

The following Example is for illustration purposes only, and is not intended to limit the scope of the present invention in any way.

Example

Toxicological Trial in the Rat

A single dose oral toxicity study in the rat was performed. The procedure used met the requirements of the limit test for acute oral toxicity described in Annex V of the EEC Commission Directive relating to the classification, packaging and labelling of dangerous substances. This is an acceptable method in the assessment of additives in animal nutrition.

The test material was a mixture of equal concentration of bacteriophages specific to Clostridium sporogenes and to Clostridium tyrobutyricum at a combined level of 2×10^{10} pfu/gram. This was administered as a single oral dose at a level of 2000 mg/kg live weight. In all cases no effects of treatment were observed and no abnormalities revealed at necropsy on termination of the study.

CLAIMS

1. The use of at least one species of bacteriophage in an area where a foodstuff is prepared or stored to treat or prevent bacterial infection in the foodstuff.

2. Use according to claim 1, wherein the foodstuff is a dairy product.

3. Use according to claim 1 or 2, wherein the foodstuff is cheese.

4. Use according to claim 3, wherein the phage is used before or following normal disinfection, before the process of blending cheeses, or before the injection of fungi.

5. Use according to any preceding claim, wherein the phage is applied as a spray.

6. Use according to any preceding claim, wherein the phage is used in concentrations of about 10^2 to about 10^{11} pfu.

7. Use according to any preceding claim wherein more than one phage variety is used.

8. Use according to any preceding claim, wherein the phage is specific for a Listeria species.

9. Use according to claim 8, wherein the Listeria species is L. monocytogenes.

10. Use according to any preceding claim, wherein the variety of phage used is changed from time to time in the said area.

Patents Act 1977
Examiner's report to the Comptroller under Section 17 (The Search Report)

Application number
9104277.0

Relevant Technical fields

(i) UK Cl (Edition K) C6F (FE; FF; FX)

(ii) Int CL (Edition 5) A01N; C12N

Search Examiner
MS N R CURTIS

Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASE: WPI; BIOTECH (DIALOG)

Date of Search
8 JUNE 1992

Documents considered relevant following a search in respect of claims 1 TO 10

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
X	EP 0414304 A2 (UNILEVER NV) see particularly claim 42	1-10

Category	Identity of document and relevant passages	Relevant to claim(s)

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family, corresponding document.

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